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(54) Title: USE OF SUBMICRON OIL–IN–WATER EMULSIONS WITH DNA VACCINES

(57) Abstract

The use of submicron oil–in–water emulsions with nucleic acid immunization techniques is disclosed. The method includes immunization with vaccine compositions containing nucleic acid molecules encoding one or more antigens of interest, as well as administration of a submicron oil–in–water adjuvant, such as MF59. The adjuvant can be administered either before, after or simultaneously with the nucleic acid vaccines.

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USE OF SUBMICRON OIL-IN-WATER EMULSIONS WITH DNA VACCINES

Technical Field

The present invention relates generally to vaccine compositions. In particular, the invention relates to the use of submicron oil-in-water emulsions with nucleic acid vaccines.

Background of the Invention

Numerous vaccine formulations which include attenuated pathogens or subunit protein antigens, have been developed. Conventional vaccine compositions often include immunological adjuvants to enhance immune responses. For example, depot adjuvants are frequently used which adsorb and/or precipitate administered antigens and which can retain the antigen at the injection site. Typical depot adjuvants include aluminum compounds and water-in-oil emulsions. However, depot adjuvants, although increasing antigenicity, often provoke severe persistent local reactions, such as granulomas, abscesses and scarring, when injected subcutaneously or intramuscularly. Other adjuvants, such as lipopolysacharrides and muramyl dipeptides, can elicit pyrogenic responses upon injection and/or Reiter's symptoms (influenza-like symptoms, generalized joint discomfort and sometimes anterior uveitis, arthritis and urethritis). Saponins, such as *Quillaja saponaria*, have also been used as immunological adjuvants in vaccine compositions against a variety of diseases. Recently, MF59, a safe, highly immunogenic, submicron oil-in-

water emulsion, has been developed for use in vaccine compositions. See, e.g., Ott et al., "MF59 -- Design and Evaluation of a Safe and Potent Adjuvant for Human Vaccines" in *Vaccine Design: The Subunit and Adjuvant Approach* (Powell, M.F. and Newman, M.J. eds.) Plenum Press, New York, 1995, pp. 277-296.

Despite the presence of such adjuvants, conventional vaccines often fail to provide adequate protection against the targeted pathogen. In this regard, there is growing evidence that vaccination against intracellular pathogens, such as a number of viruses, should target both the cellular and humoral arms of the immune system.

Cytotoxic T-lymphocytes (CTLs) play an important role in cell-mediated immune defense against intracellular pathogens such as viruses and tumor-specific antigens produced by malignant cells. CTLs mediate cytotoxicity of virally infected cells by recognizing viral determinants in conjunction with class I MHC molecules displayed by the infected cells. Cytoplasmic expression of proteins is a prerequisite for class I MHC processing and presentation of antigenic peptides to CTLs. However, immunization with killed or attenuated viruses often fails to produce the CTLs necessary to curb intracellular infection. Furthermore, conventional vaccination techniques against viruses displaying marked genetic heterogeneity and/or rapid mutation rates that facilitate selection of immune escape variants, such as HIV or influenza, are problematic. Accordingly, alternative techniques for vaccination have been developed.

Direct injection of DNA and mRNA into mammalian tissue for the purpose of eliciting an immune response has been described. See, e.g., U.S. Patent No. 5,589,466. The method, termed "nucleic

acid immunization" herein, has been shown to elicit both humoral and cell-mediated immune responses. For example, sera from mice immunized with a human immunodeficiency virus type 1 (HIV-1) DNA construct encoding the envelope glycoprotein, gp160, were shown to react with recombinant gp160 in immunoassays and lymphocytes from the injected mice were shown to proliferate in response to recombinant gp120. Wang et al., *Proc. Natl. Acad. Sci. USA* (1993) 90:4156-4160. Similarly, mice immunized with a plasmid containing a genomic copy of the human growth hormone (hGH) gene, demonstrated an antibody-based immune response. Tang et al., *Nature* (1992) 356:152-154. Intramuscular injection of DNA encoding influenza nucleoprotein driven by a mammalian promoter has been shown to elicit a CD8+ CTL response that can protect mice against subsequent lethal challenge with virus. Ulmer et al., *Science* (1993) 259:1745-1749. Immunohistochemical studies of the injection site revealed that the DNA was taken up by myeloblasts, and cytoplasmic production of viral protein could be demonstrated for at least six months.

Conventional uses of adjuvants have involved codelivery of the adjuvants with antigens, in order to invoke immune responses against the antigens. Recently, the saponin adjuvant, QS-21, has been reported to increase the cell-mediated immune response to a naked DNA vaccine directed against HIV. *Genetic Engineering News*, June 1, 1997, p. 33.

However, the use of other adjuvants, including submicron oil-in-water emulsions, to enhance immunogenicity of nucleic acid vaccines has not heretofore been described. Furthermore, the temporal dissociation between delivery of adjuvant and delivery of nucleic acid vaccines has not previously been described.

Disclosure of the Invention

The present invention is based on the surprising and unexpected discovery that the use of a submicron oil-in-water emulsion serves to enhance the immunogenicity of nucleic acid vaccines. The use of such emulsions provides a safe and effective approach for enhancing the immunogenicity of nucleic acid vaccines against a wide variety of pathogens. The submicron oil-in-water emulsion need not be administered at the same time as the gene of interest, but may be administered prior or subsequent to delivery of the gene. Indeed, surprisingly good results are seen when the emulsion is administered prior to delivery of the gene.

Accordingly, in one embodiment, the invention is directed to a method of immunization which comprises administering a submicron oil-in-water emulsion to a vertebrate subject, and transfecting cells of said subject with a recombinant vector comprising a nucleic acid molecule encoding an antigen of interest, under conditions that permit the expression of said antigen, thereby eliciting an immunological response to said antigen of interest.

In additional embodiments, the recombinant vector is a nonviral vector, or a viral vector, such as a retroviral, vaccinia or canarypox vector.

In still another embodiment, the invention is directed to a method of immunization which comprises administering MF59 to a mammalian subject and immunizing said subject with a recombinant vector comprising a nucleic acid molecule encoding a viral antigen of interest, under conditions that permit the expression of said antigen, thereby eliciting an immunological response to said antigen of interest.

These and other embodiments of the present invention will readily occur to those of ordinary skill in the art in view of the disclosure herein.

Brief Description of the Figures

Figures 1A and 1B show the results of a ^{51}Cr release assay performed on splenocytes from C3H mice given the specified adjuvant two days prior to retroviral vector delivery, as described in Example 2a. Figure 1A depicts results from mice administered undiluted retrovirus vector 6A3. Figure 1B depicts results from mice administered retrovirus vector 6A3, diluted 1:10.

Figure 2 shows the average IgG1 response to gp120 in mice pretreated with the specified adjuvant two days prior to retroviral vector delivery, as described in Example 3.

Detailed Description of the Invention

The practice of the present invention will employ, unless otherwise indicated, conventional methods of virology, microbiology, molecular biology, recombinant DNA techniques and immunology within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, et al., *Molecular Cloning: A Laboratory Manual* (2nd Edition, 1989); *DNA Cloning: A Practical Approach*, vol. I & II (D. Glover, ed.); *Oligonucleotide Synthesis* (N. Gait, ed., 1984); *A Practical Guide to Molecular Cloning* (1984); *Fundamental Virology*, 2nd Edition, vol. I & II (B.N. Fields and D.M. Knipe, eds.); *Methods In Enzymology* (S. Colowick and N. Kaplan, eds., Academic Press, Inc.); and *Handbook of Experimental Immunology*, Vols. I-IV (D.M. Weir and C.C. Blackwell, eds., 1986, Blackwell Scientific Publications)

As used in this specification and the appended claims, the singular forms "a," "an" and "the" include plural references unless the content clearly dictates otherwise.

I. Definitions

In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

By "nucleic acid immunization" is meant the introduction of a nucleic acid molecule encoding one or more selected antigens into a host cell, for the *in vivo* expression of the antigen or antigens. The nucleic acid molecule can be introduced into the recipient subject, using nonviral vectors, viral vectors or bacterial vectors (as described further below) such as by injection, inhalation, oral, intranasal and mucosal administration, or the like, or can be introduced *ex vivo*, into cells which have been removed from the host. In the latter case, the transformed cells are reintroduced into the subject where an immune response can be mounted against the antigen encoded by the nucleic acid molecule.

By "antigen" is meant a molecule which contains one or more epitopes that will stimulate a host's immune system to make a cellular antigen-specific immune response when the antigen is presented, or a humoral antibody response. Normally, an epitope will include between about 3-15, generally about 5-15, amino acids. For purposes of the present invention, antigens can be derived from any of several known viruses, bacteria, parasites and fungi. The term also intends any of the various tumor antigens. Furthermore, for purposes of the present invention, an "antigen" refers to a protein which includes modifications, such as deletions, additions and

substitutions (generally conservative in nature), to the native sequence, so long as the protein maintains the ability to elicit an immunological response. These modifications may be deliberate, as through site-directed mutagenesis, or may be accidental, such as through mutations of hosts which produce the antigens.

An "immunological response" to an antigen or composition is the development in a subject of a humoral and/or a cellular immune response to molecules present in the composition of interest. For purposes of the present invention, a "humoral immune response" refers to an immune response mediated by antibody molecules, while a "cellular immune response" is one mediated by T-lymphocytes and/or other white blood cells. One important aspect of cellular immunity involves an antigen-specific response by cytolytic T-cells ("CTL"s). CTLs have specificity for peptide antigens that are presented in association with proteins encoded by the major histocompatibility complex (MHC) and expressed on the surfaces of cells. CTLs help induce and promote the intracellular destruction of intracellular microbes, or the lysis of cells infected with such microbes. Another aspect of cellular immunity involves an antigen-specific response by helper T-cells. Helper T-cells act to help stimulate the function, and focus the activity of, nonspecific effector cells against cells displaying peptide antigens in association with MHC molecules on their surface. A "cellular immune response" also refers to the production of cytokines, chemokines and other such molecules produced by activated T-cells and/or other white blood cells, including those derived from CD4+ and CD8+ T-cells.

A composition or vaccine that elicits a cellular immune response may serve to sensitize a

vertebrate subject by the presentation of antigen in association with MHC molecules at the cell surface. The cell-mediated immune response is directed at, or near, cells presenting antigen at their surface. In addition, antigen-specific T-lymphocytes can be generated to allow for the future protection of an immunized host.

The ability of a particular antigen or composition to stimulate a cell-mediated immunological response may be determined by a number of assays, such as by lymphoproliferation (lymphocyte activation) assays, CTL cytotoxic cell assays, or by assaying for T-lymphocytes specific for the antigen in a sensitized subject. Such assays are well known in the art. See, e.g., Erickson et al., *J. Immunol.* (1993) 151:4189-4199; Doe et al., *Eur. J. Immunol.* (1994) 24:2369-2376; and the examples below.

Thus, an immunological response as used herein may be one which stimulates the production of CTLs, and/or the production or activation of helper T-cells. The antigen of interest may also elicit an antibody-mediated immune response. Hence, an immunological response may include one or more of the following effects: the production of antibodies by B-cells; and/or the activation of suppressor T-cells and/or $\gamma\delta$ T-cells directed specifically to an antigen or antigens present in the composition or vaccine of interest. These responses may serve to neutralize infectivity, and/or mediate antibody-complement, or antibody dependent cell cytotoxicity (ADCC) to provide protection to an immunized host. Such responses can be determined using standard immunoassays and neutralization assays, well known in the art.

A "coding sequence" or a sequence which "encodes" a selected antigen, is a nucleic acid molecule which is transcribed (in the case of DNA) and

translated (in the case of mRNA) into a polypeptide *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus. A coding sequence can include, but is not limited to, cDNA from viral, procaryotic or eucaryotic mRNA, genomic DNA sequences from viral or procaryotic DNA, and even synthetic DNA sequences. A transcription termination sequence may be located 3' to the coding sequence.

A "nucleic acid" molecule can include, but is not limited to, procaryotic sequences, eucaryotic mRNA, cDNA from eucaryotic mRNA, genomic DNA sequences from eucaryotic (e.g., mammalian) DNA, and even synthetic DNA sequences. The term also captures sequences that include any of the known base analogs of DNA and RNA.

By "vector" is meant any genetic element, such as a plasmid, phage, transposon, cosmid, chromosome, virus, virion, recombinant virus, etc., which can deliver gene sequences to a desired cell or tissue. Thus, the term includes cloning and expression vehicles, as well as viral vectors.

"Operably linked" refers to an arrangement of elements wherein the components so described are configured so as to perform their usual function. Thus, a given promoter operably linked to a coding sequence is capable of effecting the expression of the coding sequence when the proper enzymes are present. The promoter need not be contiguous with the coding sequence, so long as it functions to direct the expression thereof. Thus, for example, intervening untranslated yet transcribed sequences can be present between the promoter sequence and the coding sequence

and the promoter sequence can still be considered "operably linked" to the coding sequence.

"Recombinant" as used herein to describe a nucleic acid molecule means a polynucleotide of genomic, cDNA, semisynthetic, or synthetic origin which, by virtue of its origin or manipulation: (1) is not associated with all or a portion of the polynucleotide with which it is associated in nature; and/or (2) is linked to a polynucleotide other than that to which it is linked in nature. The term "recombinant" as used with respect to a protein or polypeptide means a polypeptide produced by expression of a recombinant polynucleotide.

Two nucleic acid or polypeptide sequences are "substantially homologous" when at least about 70%, preferably at least about 80-90%, and most preferably at least about 95%, of the nucleotides or amino acids match over a defined length of the molecule. As used herein, substantially homologous also refers to sequences showing identity to the specified nucleic acid or polypeptide sequence. Nucleic acid sequences that are substantially homologous can be identified in a Southern hybridization experiment under, for example, stringent conditions, as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Sambrook et al., *supra*; *DNA Cloning*, vols I & II, supra; *Nucleic Acid Hybridization*, *supra*. Such sequences can also be confirmed and further characterized by direct sequencing of PCR products.

The terms "effective amount" or "pharmaceutically effective amount" of an agent, as provided herein, refer to a nontoxic but sufficient amount of the agent to provide the desired immunological response and corresponding therapeutic

effect. As will be pointed out below, the exact amount required will vary from subject to subject, depending on the species, age, and general condition of the subject, the severity of the condition being treated, and the particular antigen of interest, mode of administration, and the like. An appropriate "effective" amount in any individual case may be determined by one of ordinary skill in the art using routine experimentation.

As used herein, "treatment" refers to any of (i) the prevention of infection or reinfection, as in a traditional vaccine, (ii) the reduction or elimination of symptoms, and (iii) the substantial or complete elimination of the pathogen in question from the patient. Treatment may be effected prophylactically (prior to infection) or therapeutically (following infection).

By "vertebrate subject" is meant any member of the subphylum cordata, including, without limitation, humans and other primates, including non-human primates such as chimpanzees and other apes and monkey species; farm animals such as cattle, sheep, pigs, goats and horses; domestic mammals such as dogs and cats; laboratory animals including rodents such as mice, rats and guinea pigs; birds, including domestic, wild and game birds such as chickens, turkeys and other gallinaceous birds, ducks, geese, and the like. The term does not denote a particular age. Thus, both adult and newborn individuals are intended to be covered. The system described above is intended for use in any of the above vertebrate species, since the immune systems of all of these vertebrates operate similarly.

II. Modes of Carrying Out the Invention

The present invention is based on the discovery that the use of submicron oil-in-water emulsions in combination with nucleic acid immunization, can provide a vigorous immune response, even when the gene delivered encodes for a protein which is by itself weakly immunogenic.

In particular, the method of the invention provides for cell-mediated immunity, and/or humoral antibody responses. Thus, in addition to a conventional antibody response, the system herein described can provide for, e.g., the association of the expressed antigens with class I MHC molecules such that an *in vivo* cellular immune response to the antigen of interest can be mounted which stimulates the production of CTLs to allow for future recognition of the antigen. Furthermore, the method may elicit an antigen-specific response by helper T-cells. Accordingly, the methods of the present invention will find use with any antigen for which cellular and/or humoral immune responses are desired, including antigens derived from viral, bacterial, fungal and parasitic pathogens that may induce antibodies, T-cell helper epitopes and T-cell cytotoxic epitopes. Such antigens include, but are not limited to, those encoded by human and animal viruses and can correspond to either structural or non-structural proteins.

The technique is particularly useful for immunization against intracellular viruses and tumor cell antigens which normally elicit poor immune responses. For example, the present invention will find use for stimulating an immune response against a wide variety of proteins from the herpesvirus family, including proteins derived from herpes simplex virus (HSV) types 1 and 2, such as HSV-1 and HSV-2 glycoproteins gB, gD and gH; antigens derived from

varicella zoster virus (VZV), Epstein-Barr virus (EBV) and cytomegalovirus (CMV) including CMV gB and gH; and antigens derived from other human herpesviruses such as HHV6 and HHV7. (See, e.g. Chee et al., *Cytomegaloviruses* (J.K. McDougall, ed., Springer-Verlag 1990) pp. 125-169, for a review of the protein coding content of cytomegalovirus; McGeoch et al., *J. Gen. Virol.* (1988) 69:1531-1574, for a discussion of the various HSV-1 encoded proteins; U.S. Patent No. 5,171,568 for a discussion of HSV-1 and HSV-2 gB and gD proteins and the genes encoding therefor; Baer et al., *Nature* (1984) 310:207-211, for the identification of protein coding sequences in an EBV genome; and Davison and Scott, *J. Gen. Virol.* (1986) 67:1759-1816, for a review of VZV.)

Polynucleotide sequences encoding antigens from the hepatitis family of viruses, including hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), the delta hepatitis virus (HDV), hepatitis E virus (HEV) and hepatitis G virus (HGV), can also be conveniently used in the techniques described herein. By way of example, the viral genomic sequence of HCV is known, as are methods for obtaining the sequence. See, e.g., International Publication Nos. WO 89/04669; WO 90/11089; and WO 90/14436. The HCV genome encodes several viral proteins, including E1 (also known as E) and E2 (also known as E2/NSI) and an N-terminal nucleocapsid protein (termed "core") (see, Houghton et al., *Hepatology* (1991) 14:381-388, for a discussion of HCV proteins, including E1 and E2). The sequences encoding each of these proteins, as well as antigenic fragments thereof, will find use in the present methods. Similarly, the coding sequence for the δ-antigen from HDV is known (see, e.g., U.S. Patent No. 5,378,814) and this sequence can also be conveniently

used in the present methods. Additionally, antigens derived from HBV, such as the core antigen, the surface antigen, sAg, as well as the presurface sequences, pre-S1 and pre-S2 (formerly called pre-S), as well as combinations of the above, such as sAg/pre-S1, sAg/pre-S2, sAg/pre-S1/pre-S2, and pre-S1/pre-S2, will find use herein. See, e.g., "HBV Vaccines - from the laboratory to license: a case study" in Mackett, M. and Williamson, J.D., *Human Vaccines and Vaccination*, pp. 159-176, for a discussion of HBV structure; and U.S. Patent Nos. 4,722,840, 5,098,704, 5,324,513; Beames et al., *J. Virol.* (1995) 69:6833-6838, Birnbaum et al., *J. Virol.* (1990) 64:3319-3330; and Zhou et al., *J. Virol.* (1991) 65:5457-5464.

Polynucleotide sequences encoding antigens derived from other viruses will also find use in the claimed methods, such as without limitation, proteins from members of the families Picornaviridae (e.g., polioviruses, etc.); Caliciviridae; Togaviridae (e.g., rubella virus, dengue virus, etc.); Flaviviridae; Coronaviridae; Reoviridae; Birnaviridae; Rhabodoviridae (e.g., rabies virus, etc.); Filoviridae; Paramyxoviridae (e.g., mumps virus, measles virus, respiratory syncytial virus, etc.); Orthomyxoviridae (e.g., influenza virus types A, B and C, etc.); Bunyaviridae; Arenaviridae; Retroviradae (e.g., HTLV-I; HTLV-II; HIV-1 (also known as HTLV-III, LAV, ARV, hTLR, etc.)), including but not limited to antigens from the isolates HIV_{IIIb}, HIV_{SF2}, HIV_{LAV}, HIV_{LAI}, HIV_{MN}); HIV-1_{CM235}, HIV-1_{US4}; HIV-2; simian immunodeficiency virus (SIV) among others. Additionally, antigens may also be derived from human papillomavirus (HPV) and the tick-borne encephalitis viruses. See, e.g. Virology, 3rd Edition (W.K. Joklik ed. 1988); *Fundamental Virology*, 2nd Edition (B.N.

Fields and D.M. Knipe, eds. 1991), for a description of these and other viruses.

More particularly, genes encoding the gp120 envelope protein from any of the above HIV isolates, including members of the various genetic subtypes of HIV, are known and reported (see, e.g., Myers et al., Los Alamos Database, Los Alamos National Laboratory, Los Alamos, New Mexico (1992); Myers et al., *Human Retroviruses and Aids, 1990*, Los Alamos, New Mexico: Los Alamos National Laboratory; and Modrow et al., *J. Virol.* (1987) 61:570-578, for a comparison of the envelope gene sequences of a variety of HIV isolates) and sequences derived from any of these isolates will find use in the present methods. Furthermore, the invention is equally applicable to other immunogenic proteins derived from any of the various HIV isolates, including any of the various envelope proteins such as gp160 and gp41, gag antigens such as p24gag and p55gag, as well as proteins derived from the pol region.

As explained above, influenza virus is another example of a virus for which the present invention will be particularly useful. Specifically, the envelope glycoproteins HA and NA of influenza A are of particular interest for generating an immune response. Numerous HA subtypes of influenza A have been identified (Kawaoka et al., *Virology* (1990) 179:759-767; Webster et al., "Antigenic variation among type A influenza viruses," p. 127-168. In: P. Palese and D.W. Kingsbury (ed.), *Genetics of influenza viruses*. Springer-Verlag, New York). Thus, the gene sequences encoding proteins derived from any of these isolates can also be used in the nucleic acid immunization techniques described herein.

The techniques can be used for the delivery of discrete antigens, larger portions of the genome in

question and, for example, a proviral DNA which includes nearly all of the viral genome.

The methods described herein will also find use with DNA sequences encoding numerous bacterial antigens, such as those derived from organisms that cause diphtheria, cholera, tuberculosis, tetanus, pertussis, meningitis, and other pathogenic states, including, without limitation, *Meningococcus* A, B and C, *Hemophilus influenza* type B (HIB), and *Helicobacter pylori*. Examples of parasitic antigens include those derived from organisms causing malaria and Lyme disease.

Furthermore, the methods described herein provide a means for treating a variety of malignant cancers. For example, the system of the present invention can be used to mount both humoral and cell-mediated immune responses to particular proteins specific to the cancer in question, such as an activated oncogene, a fetal antigen, or an activation marker. Such tumor antigens include any of the various MAGEs (melanoma associated antigen E), including MAGE 1, 2, 3, 4, etc. (Boon, T. *Scientific American* (March 1993):82-89); any of the various tyrosinases; MART 1 (melanoma antigen recognized by T cells), mutant ras; mutant p53; p97 melanoma antigen; CEA (carcinoembryonic antigen), among others.

It is readily apparent that the subject invention can be used to prevent or treat a wide variety of diseases.

Polynucleotide sequences coding for the above-described molecules can be obtained using recombinant methods, such as by screening cDNA and genomic libraries from cells expressing the gene, or by deriving the gene from a vector known to include the same. Furthermore, the desired gene can be isolated directly from cells and tissues containing

the same, using standard techniques, such as phenol extraction and PCR of cDNA or genomic DNA. See, e.g., Sambrook et al., *supra*, for a description of techniques used to obtain and isolate DNA. The gene of interest can also be produced synthetically, rather than cloned. The nucleotide sequence can be designed with the appropriate codons for the particular amino acid sequence desired. In general, one will select preferred codons for the intended host in which the sequence will be expressed. The complete sequence is assembled from overlapping oligonucleotides prepared by standard methods and assembled into a complete coding sequence. See, e.g., Edge, *Nature* (1981) 292:756; Nambair et al., *Science* (1984) 223:1299; Jay et al., *J. Biol. Chem.* (1984) 259:6311.

Next, the gene sequence encoding the desired antigen can be inserted into a vector which includes control sequences operably linked to the desired coding sequence, which allow for the expression of the gene *in vivo* in the subject species. For example, typical promoters for mammalian cell expression include the SV40 early promoter, a CMV promoter such as the CMV immediate early promoter (Chapman et al., *Nucl. Acids Res.* (1991) 19:3979-3986), the mouse mammary tumor virus LTR promoter, the adenovirus major late promoter (Ad MLP), and the herpes simplex virus promoter, among others. Other nonviral promoters, such as a promoter derived from the murine metallothionein gene, will also find use for mammalian expression. Typically, transcription termination and polyadenylation sequences will also be present, located 3′ to the translation stop codon. Preferably, a sequence for optimization of initiation of translation, located 5′ to the coding sequence, is also present. Examples of transcription terminator/polyadenylation signals include those

derived from SV40, as described in Sambrook et al., *supra*, as well as a bovine growth hormone terminator sequence. Introns, containing splice donor and acceptor sites, may also be designed into the constructs for use with the present invention.

Enhancer elements may also be used herein to increase expression levels of the mammalian constructs. Examples include the SV40 early gene enhancer, as described in Dijkema et al., *EMBO J.* (1985) 4:761, the enhancer/promoter derived from the long terminal repeat (LTR) of the Rous Sarcoma Virus, as described in Gorman et al., *Proc. Natl. Acad. Sci. USA* (1982b) 79:6777 and elements derived from human CMV, as described in Boshart et al., *Cell* (1985) 41:521, such as elements included in the CMV intron A sequence.

Furthermore, plasmids can be constructed which include a chimeric gene sequence, encoding e.g., multiple antigens of interest, for example derived from more than one viral isolate. Additionally, genes coding for immune modulating agents which can enhance antigen presentation, attract lymphocytes to the site of gene expression or promote expansion of the population of lymphocytes to the site of gene expression or promote expansion of the population of lymphocytes which respond to the expressed antigen, can also be present. Such agents include cytokines, lymphokines, and chemokines, including but not limited to IL-2, modified IL-2 (cys125→ser125), GM-CSF, IL-12, γ-interferon, IP-10, MIP1α, MIP1β and RANTES. Additionally, immune molecules such as TAP transporters, costimulatory molecules such as B7, β2M, class I or II MHC genes (syngeneic or allogeneic), and other genes coding for proteins that are required for efficient immune responses but are not expressed due to specific inhibition or deletion, will also find use

in the constructs. This is particularly relevant in tumor cells and in some infected cells where antigen presentation is often reduced.

The above sequences can be administered using separate vectors or can be present on the vector bearing the gene encoding the antigen of interest. If present on the same vector, the additional gene sequences can either precede or follow the gene encoding the antigen of interest in a dicistronic gene configuration. Additional control elements can be situated between the various genes for efficient translation of RNA from the distal coding region. Alternatively, a chimeric transcription unit having a single open reading frame encoding both the gene of interest and the modulator, can also be constructed. Either a fusion can be made to allow for the synthesis of a chimeric protein or alternatively, protein processing signals can be engineered to provide cleavage by a protease such as a signal peptidase, thus allowing liberation of the two or more proteins derived from translation of the template RNA. Such signals for processing of a polyprotein exist in, e.g., flaviviruses, pestiviruses such as HCV, and picornaviruses, and can be engineered into the constructs. The processing protease may also be expressed in this system either independently or as part of a chimera with the antigen and/or cytokine coding region(s). The protease itself can be both a processing enzyme and a vaccine antigen.

Once complete, the constructs are used for nucleic acid immunization using standard gene delivery protocols. Methods for gene delivery are known in the art. See, e.g., U.S. Patent Nos. 5,399,346, 5,580,859, 5,589,466. Genes can be delivered either directly to the vertebrate subject or, alternatively, delivered *ex vivo*, to cells derived from the subject

and the cells reimplanted in the subject. Genes can be delivered using nonviral vectors, as described above, viral vectors or bacterial vectors.

A number of viral based systems have been developed for gene transfer into mammalian cells. For example, retroviruses provide a convenient platform for gene delivery systems. A selected gene can be inserted into a vector and packaged in retroviral particles using techniques known in the art. The recombinant virus can then be isolated and delivered to cells of the subject either *in vivo* or *ex vivo*. A number of retroviral systems have been described (see, e.g., U.S. Patent No. 5,219,740; International Publication Nos. WO 91/02805 and WO 93/15207; Miller and Rosman, *BioTechniques* (1989) 7:980-990; Miller, A.D., *Human Gene Therapy* (1990) 1:5-14; Scarpa et al., *Virology* (1991) 180:849-852; Burns et al., *Proc. Natl. Acad. Sci. USA* (1993) 90:8033-8037; and Boris-Lawrie and Temin, *Cur. Opin. Genet. Develop.* (1993) 3:102-109).

A number of adenovirus vectors have also been described. Unlike retroviruses which integrate into the host genome, adenoviruses persist extrachromosomally thus minimizing the risks associated with insertional mutagenesis (Haj-Ahmad and Graham, *J. Virol.* (1986) 57:267-274; Bett et al., *J. Virol.* (1993) 67:5911-5921; Mittereder et al., *Human Gene Therapy* (1994) 5:717-729; Seth et al., *J. Virol.* (1994) 68:933-940; Barr et al., *Gene Therapy* (1994) 1:51-58; Berkner, K.L. *BioTechniques* (1988) 6:616-629; and Rich et al., *Human Gene Therapy* (1993) 4:461-476).

Additionally, various adeno-associated virus (AAV) vector systems have been developed for gene delivery. AAV vectors can be readily constructed using techniques well known in the art. See, e.g., U.S. Patent Nos. 5,173,414 and 5,139,941;

International Publication Nos. WO 92/01070 (published 23 January 1992) and WO 93/03769 (published 4 March 1993); Lebkowski et al., *Molec. Cell. Biol.* (1988) 8:3988-3996; Vincent et al., *Vaccines 90* (1990) (Cold Spring Harbor Laboratory Press); Carter, B.J. *Current Opinion in Biotechnology* (1992) 3:533-539; Muzyczka, N. *Current Topics in Microbiol. and Immunol.* (1992) 158:97-129; Kotin, R.M. *Human Gene Therapy* (1994) 5:793-801; Shelling and Smith, *Gene Therapy* (1994) 1:165-169; and Zhou et al., *J. Exp. Med.* (1994) 179:1867-1875.

Additional viral vectors which will find use for delivering the nucleic acid molecules encoding the antigens of interest include those derived from the pox family of viruses, including vaccinia virus and avian poxvirus. By way of example, vaccinia virus recombinants expressing the genes can be constructed as follows. The DNA encoding the particular antigen is first inserted into an appropriate vector so that it is adjacent to a vaccinia promoter and flanking vaccinia DNA sequences, such as the sequence encoding thymidine kinase (TK). This vector is then used to transfect cells which are simultaneously infected with vaccinia. Homologous recombination serves to insert the vaccinia promoter plus the gene encoding the antigen of interest into the viral genome. The resulting TK^- recombinant can be selected by culturing the cells in the presence of 5-bromodeoxyuridine and picking viral plaques resistant thereto.

Alternatively, avipoxviruses, such as the fowlpox and canarypox viruses, can also be used to deliver the genes. Recombinant avipox viruses, expressing immunogens from mammalian pathogens, are known to confer protective immunity when administered to non-avian species. The use of an avipox vector is particularly desirable in human and other mammalian

species since members of the avipox genus can only productively replicate in susceptible avian species and therefore are not infective in mammalian cells. Methods for producing recombinant avipoxviruses are known in the art and employ genetic recombination, as described above with respect to the production of vaccinia viruses. See, e.g., WO 91/12882; WO 89/03429; and WO 92/03545.

Molecular conjugate vectors, such as the adenovirus chimeric vectors described in Michael et al., *J. Biol. Chem.* (1993) 268:6866-6869 and Wagner et al., *Proc. Natl. Acad. Sci. USA* (1992) 89:6099-6103, can also be used for gene delivery. Additionally, the gene of interest can be delivered using pseudovirions, such as a noninfectious retrovirus-like particle, described in e.g., International Publication No. WO 91/05864, published 2 May 1991.

Members of the Alphavirus genus, such as but not limited to vectors derived from the Sindbis and Semliki Forest viruses, will also find use as viral vectors for delivering the gene of interest. For a description of Sinbus-virus derived vectors useful for the practice of the instant methods, see, International Publication No. WO 95/07995.

A vaccinia based infection/transfection system can be conveniently used to provide for inducible, transient expression of the gene of interest in a host cell. In this system, cells are first infected *in vitro* with a vaccinia virus recombinant that encodes the bacteriophage T7 RNA polymerase. This polymerase displays exquisite specificity in that it only transcribes templates bearing T7 promoters. Following infection, cells are transfected with the polynucleotide of interest, driven by a T7 promoter. The polymerase expressed in the cytoplasm from the vaccinia virus recombinant

transcribes the transfected DNA into RNA which is then translated into protein by the host translational machinery. The method provides for high level, transient, cytoplasmic production of large quantities of RNA and its translation products. See, e.g., Elroy-Stein and Moss, *Proc. Natl. Acad. Sci. USA* (1990) 87:6743-6747; Fuerst et al., *Proc. Natl. Acad. Sci. USA* (1986) 83:8122-8126.

As an alternative approach to infection with vaccinia or avipox virus recombinants, or to the delivery of genes using other viral vectors, an amplification system can be used that will lead to high level expression following introduction into host cells. Specifically, a T7 RNA polymerase promoter preceding the coding region for T7 RNA polymerase can be engineered. Translation of RNA derived from this template will generate T7 RNA polymerase which in turn will transcribe more template. Concomitantly, there will be a cDNA whose expression is under the control of the T7 promoter. Thus, some of the T7 RNA polymerase generated from translation of the amplification template RNA will lead to transcription of the desired gene. Because some T7 RNA polymerase is required to initiate the amplification, T7 RNA polymerase can be introduced into cells along with the template(s) to prime the transcription reaction. The polymerase can be introduced as a protein or on a plasmid encoding the RNA polymerase. For a further discussion of T7 systems and their use for transforming cells, see, e.g., International Publication No. WO 94/26911; Studier and Moffatt, *J. Mol. Biol.* (1986) 189:113-130; Deng and Wolff, *Gene* (1994) 143:245-249; Gao et al., *Biochem. Biophys. Res. Commun.* (1994) 200:1201-1206; Gao and Huang, *Nuc. Acids Res.* (1993) 21:2867-2872; Chen et al., *Nuc.*

Acids Res. (1994) 22:2114-2120; and U.S. Patent No. 5,135,855.

Bacterial vectors may also be used to deliver the gene of interest, such as but not limited to vectors derived from *Mycobacteria*, such as *M. smegmatis* and *M. bovis* bacillus Calmette-Guèrin (BCG) (see, e.g., Stover et al., *Nature* (1991) 351:456 and Aldovini and Young, *Nature* (1991) 351:479); *Salmonella*-derived vectors, such as attenuated mutants of *S. typhimurium*, *S. sobrinus* and *S. dublin* (see, e.g., Cárdenas and Clements, *Vaccine* (1993) 11:126 and Schödel et al., *Vaccine* (1993) 11:143); as well as vectors derived from *E. coli*; *Listeria*, such as *L. monocytogenes* (see, e.g., Frankel et al., *J. Immunol.* (1995) 155:4775); *Shigella*, and the like.

The gene of interest can also be packaged in liposomes prior to delivery to the subject or to cells derived therefrom, with or without the accompanying antigen. Lipid encapsulation is generally accomplished using liposomes which are able to stably bind or entrap and retain nucleic acid. The ratio of condensed DNA to lipid preparation can vary but will generally be around 1:1 (mg DNA:micromoles lipid), or more of lipid. For a review of the use of liposomes as carriers for delivery of nucleic acids, see, Hug and Sleight, *Biochim. Biophys. Acta.* (1991) 1097:1-17; Straubinger et al., in *Methods of Enzymology* (1983), Vol. 101, pp. 512-527.

Liposomal preparations for use in the instant invention include cationic (positively charged), anionic (negatively charged) and neutral preparations, with cationic liposomes particularly preferred. Cationic liposomes have been shown to mediate intracellular delivery of plasmid DNA (Felgner et al., *Proc. Natl. Acad. Sci. USA* (1987) 84:7413-7416); mRNA (Malone et al., *Proc. Natl. Acad. Sci. USA*

(1989) 86:6077-6081); and purified transcription factors (Debs et al., *J. Biol. Chem.* (1990) 265:10189-10192), in functional form.

Cationic liposomes are readily available. For example, N[1-2,3-dioleyloxy)propyl]-N,N,N-triethylammonium (DOTMA) liposomes are available under the trademark Lipofectin, from GIBCO BRL, Grand Island, NY. (See, also, Felgner et al., *Proc. Natl. Acad. Sci. USA* (1987) 84:7413-7416). Other commercially available lipids include transfectace (DDAB/DOPE) and DOTAP/DOPE (Boerhinger). Other cationic liposomes can be prepared from readily available materials using techniques well known in the art. See, e.g., Szoka et al., *Proc. Natl. Acad. Sci. USA* (1978) 75:4194-4198; PCT Publication No. WO 90/11092 for a description of the synthesis of DOTAP (1,2-bis(oleoyloxy)-3-(trimethylammonio)propane) liposomes.

Similarly, anionic and neutral liposomes are readily available, such as from Avanti Polar Lipids (Birmingham, AL), or can be easily prepared using readily available materials. Such materials include phosphatidyl choline, cholesterol, phosphatidyl ethanolamine, dioleoylphosphatidyl choline (DOPC), dioleoylphosphatidyl glycerol (DOPG), dioleoylphoshatidyl ethanolamine (DOPE), among others. These materials can also be mixed with the DOTMA and DOTAP starting materials in appropriate ratios. Methods for making liposomes using these materials are well known in the art.

The liposomes can comprise multilammelar vesicles (MLVs), small unilamellar vesicles (SUVs), or large unilamellar vesicles (LUVs). The various liposome-nucleic acid complexes are prepared using methods known in the art. See, e.g., Straubinger et al., in METHODS OF IMMUNOLOGY (1983), Vol. 101, pp.

512-527; Szoka et al., *Proc. Natl. Acad. Sci. USA* (1978) 75:4194-4198; Papahadjopoulos et al., *Biochim. Biophys. Acta* (1975) 394:483; Wilson et al., *Cell* (1979) 17:77); Deamer and Bangham, *Biochim. Biophys. Acta* (1976) 443:629; Ostro et al., *Biochem. Biophys. Res. Commun.* (1977) 76:836; Fraley et al., *Proc. Natl. Acad. Sci. USA* (1979) 76:3348); Enoch and Strittmatter, *Proc. Natl. Acad. Sci. USA* (1979) 76:145); Fraley et al., *J. Biol. Chem.* (1980) 255:10431; Szoka and Papahadjopoulos, *Proc. Natl. Acad. Sci. USA* (1978) 75:145; and Schaefer-Ridder et al., *Science* (1982) 215:166.

The DNA and/or protein antigen(s) can also be delivered in cochleate lipid compositions similar to those described by Papahadjopoulos et al., *Biochem. Biophys. Acta.* (1975) 394:483-491. See, also, U.S. Patent Nos. 4,663,161 and 4,871,488.

Particulate systems and polymers can be used for the *in vivo* or *ex vivo* delivery of the gene of interest. For example, polymers such as polylysine, polyarginine, polyornithine, spermine, spermidine, as well as conjugates of these molecules, are useful for transferring a nucleic acid of interest. Similarly, DEAE dextran-mediated transfection, calcium phosphate precipitation or precipitation using other insoluble inorganic salts, such as strontium phosphate, aluminum silicates including bentonite and kaolin, chromic oxide, magnesium silicate, talc, and the like, will find use with the present methods. See, e.g., Felgner, P.L., *Advanced Drug Delivery Reviews* (1990) 5:163-187, for a review of delivery systems useful for gene transfer.

Additionally, biolistic delivery systems employing particulate carriers such as gold and tungsten, are especially useful for delivering genes of interest. The particles are coated with the gene

to be delivered and accelerated to high velocity, generally under a reduced atmosphere, using a gun powder discharge from a "gene gun." For a description of such techniques, and apparatuses useful therefore, see, e.g., U.S. Patent Nos. 4,945,050; 5,036,006; 5,100,792; 5,179,022; 5,371,015; and 5,478,744.

The recombinant vectors (with or without associated lipids or carriers) are formulated into compositions for delivery to the vertebrate subject. These compositions may either be prophylactic (to prevent infection) or therapeutic (to treat disease after infection). The compositions will comprise a "therapeutically effective amount" of the gene of interest such that an amount of the antigen can be produced *in vivo* so that an immune response is generated in the individual to which it is administered. The exact amount necessary will vary depending on the subject being treated; the age and general condition of the subject to be treated; the capacity of the subject's immune system to synthesize antibodies; the degree of protection desired; the severity of the condition being treated; the particular antigen selected and its mode of administration, among other factors. An appropriate effective amount can be readily determined by one of skill in the art. Thus, a "therapeutically effective amount" will fall in a relatively broad range that can be determined through routine trials. For example, for purposes of the present invention, an effective dose will typically range from about 1 μg to about 100 mg, more preferably from about 10 μg to about 1 mg, of the DNA constructs.

The compositions will generally include one or more "pharmaceutically acceptable excipients or vehicles" such as water, saline, glycerol, polyethyleneglycol, hyaluronic acid, ethanol, etc.

Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present in such vehicles. Certain facilitators of nucleic acid uptake and/or expression can also be included in the compositions or coadministered, such as, but not limited to, bupivacaine, cardiotoxin and sucrose.

Once formulated, the compositions of the invention can be administered directly to the subject or, alternatively, delivered *ex vivo*, to cells derived from the subject, using methods such as those described above. For example, methods for the *ex vivo* delivery and reimplantation of transformed cells into a subject are known in the art and will include e.g., dextran-mediated transfection, calcium phosphate precipitation, polybrene mediated transfection, lipofectamine and LT-1 mediated transfection, protoplast fusion, electroporation, encapsulation of the polynucleotide(s) (with or without the corresponding antigen) in liposomes, and direct microinjection of the DNA into nuclei.

Direct delivery of the compositions *in vivo* will generally be accomplished with or without viral vectors, as described above, by injection using either a conventional syringe or a gene gun, such as the Accell® gene delivery system (Agracetus, Inc., Middleton, WI). The constructs can be injected either subcutaneously, epidermally, intradermally, intramucosally such as nasally, rectally and vaginally, intraperitoneally, intravenously, orally or intramuscularly. For example, delivery of DNA into cells of the epidermis provides access to skin-associated lymphoid cells and provides for a transient presence of DNA in the vaccine recipient. Other modes of administration include oral and pulmonary

administration, suppositories, and transdermal applications.

Dosage treatment may be a single dose schedule or a multiple dose schedule. A multiple dose schedule is one in which a primary course of vaccination may be with 1-10 separate doses, followed by other doses given at subsequent time intervals, chosen to maintain and/or reinforce the immune response, for example at 1-4 months for a second dose, and if needed, a subsequent dose(s) after several months. The boost may be with the nucleic acid vaccines or may comprise subunit antigen compositions including the antigen encoded by the delivered nucleic acid constructs. The dosage regimen will, at least in part, be determined by the need of the subject and be dependent on the judgment of the practitioner.

If prevention of disease is desired, the vaccines are generally administered prior to primary infection with the pathogen of interest. If treatment is desired, e.g., the reduction of symptoms or recurrences, the vaccines are generally administered subsequent to primary infection.

As explained above, a submicron oil-in-water emulsion formulation will also be administered to the vertebrate subject, either prior to, concurrent with, or subsequent to, delivery of the gene. If simultaneous delivery is desired, the submicron oil-in-water formulation can be included in the nucleic acid compositions. Alternatively, and preferably, the oil-in-water emulsions are administered separately, prior to delivery of the gene, either to the same site of delivery as the nucleic acid compositions or to a different delivery site. If administered prior to nucleic acid immunization, the formulations can be administered as early as 5-10 days prior to nucleic acid immunization, preferably 3-5 days prior to

nucleic acid immunization and most preferably 1-3 or 2 days prior to immunization with the nucleic acids of interest.

Examples of suitable submicron oil-in-water formulations for use with the present invention will include nontoxic, metabolizable oils, such as vegetable oils, fish oils, animal oils or synthetically prepared oils. Fish oils, such as cod liver oil, shark liver oils and whale oils, are preferred, with squalene, 2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene, found in shark liver oil, particularly preferred. The oil component will be present in an amount of from about 0.5% to about 20% by volume, preferably in an amount up to about 15%, more preferably in an amount of from about 1% to about 12% and most preferably from 1% to about 4% oil.

The aqueous portion of the adjuvant can be buffered saline or unadulterated water. If the compositions are to be administered parenterally, it is preferable to make up the final solutions so that the tonicity, i.e., osmolality, is essentially the same as normal physiological fluids, in order to prevent post-administration swelling or rapid absorption of the composition due to differential ion concentrations between the composition and physiological fluids. If saline is used rather than water, it is preferable to buffer the saline in order to maintain a pH compatible with normal physiological conditions. Also, in certain instances, it may be necessary to maintain the pH at a particular level in order to insure the stability of certain composition components. Thus, the pH of the compositions will generally be pH 6-8 and pH can be maintained using any physiologically acceptable buffer, such as phosphate, acetate, tris, bicarbonate or carbonate buffers, or the like. The quantity of the aqueous agent present

will generally be the amount necessary to bring the composition to the desired final volume.

Emulsifying agents suitable for use in the oil-in-water formulations include, without limitation, sorbitan-based non-ionic surfactants such as those commercially available under the name of Span® or Arlacel®; polyoxyethylene sorbitan monoesters and polyoxyethylene sorbitan triesters, commercially known by the name Tween®; polyoxyethylene fatty acids available under the name Myrj®; polyoxyethylene fatty acid ethers derived from lauryl, acetyl, stearyl and oleyl alcohols, such as those known by the name of Brij®; and the like. These substances are readily available from a number of commercial sources, including ICI America's Inc., Wilmington, DE. These emulsifying agents may be used alone or in combination. The emulsifying agent will usually be present in an amount of 0.02% to about 2.5% by weight (w/w), preferably 0.05% to about 1%, and most preferably 0.01% to about 0.5. The amount present will generally be about 20-30% of the weight of the oil used. The emulsions can also contain other immunostimulating agents, such as muramyl peptides, including, but not limited to, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acteyl-normuramyl-L-alanyl-D-isogluatme (nor-MDP), N-acetylmuramyl-L-alanyl-D-isogluatminyl-L-alanine-2-(1'-2'-dipalmitoyl-*sn*-glycero-3-huydroxyphosphoryloxy)-ethylamine (MTP-PE), etc. Immunostimulating bacterial cell wall components, such as monophosphorylipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), may also be present. In order to produce submicron particles, i.e., particles less than 1 micron in diameter and in the nanometer size range, a number of techniques can be used. For example, commercial emulsifiers can be

used that operate by the principle of high shear forces developed by forcing fluids through small apertures under high pressure. Examples of commercial emulsifiers include, without limitation, Model 110Y microfluidizer (Microfluidics, Newton, MA), Gaulin Model 30CD (Gaulin, Inc., Everett, MA), and Rainnie Minilab Type 8.30H (Miro Atomizer Food and Dairy, Inc., Hudson, WI). The appropriate pressure for use with an individual emulsifier is readily determined by one of skill in the art. For example, when the Model 110Y microfluidizer is used, operation at 5000 to 30,000 psi produces oil droplets with diameters of about 100 to 750 nm.

The size of the oil droplets can be varied by changing the ratio of detergent to oil (increasing the ratio decreases droplet size), operating pressure (increasing operating pressure reduces droplet size), temperature (increasing temperature decreases droplet size), and adding an amphipathic immunostimulating agent (adding such agents decreases droplet size). Actual droplet size will vary with the particular detergent, oil and immunostimulating agent (if any) and with the particular operating conditions selected. Droplet size can be verified by use of sizing instruments, such as the commercial Sub-Micron Particle Analyzer (Model N4MD) manufactured by the Coulter Corporation, and the parameters can be varied using the guidelines set forth above until substantially all droplets are less than 1 micron in diameter, preferably less than about 0.8 microns in diameter, and most preferably less than about 0.5 microns in diameter. By substantially all is meant at least about 80% (by number), preferably at least about 90%, more preferably at least about 95%, and most preferably at least about 98%. The particle size

distribution is typically Gaussian, so that the average diameter is smaller than the stated limits.

Particularly preferred submicron oil-in-water emulsions for use herein are squalene/water emulsions optionally containing varying amounts of MTP-PE, such as the submicron oil-in-water emulsion known as "MF59" (International Publication No. WO 90/14837; Ott et al., "MF59 -- Design and Evaluation of a Safe and Potent Adjuvant for Human Vaccines" in *Vaccine Design: The Subunit and Adjuvant Approach* (Powell, M.F. and Newman, M.J. eds.) Plenum Press, New York, 1995, pp. 277-296). MF59 contains 4-5% w/v Squalene (e.g., 4.3%), 0.25-0.5% w/v Tween 80®, and 0.5% w/v Span 85® and optionally contains various amounts of MTP-PE, formulated into submicron particles using a microfluidizer such as Model 110Y microfluidizer (Microfluidics, Newton, MA). For example, MTP-PE may be present in an amount of about 0-500 μg/dose, more preferably 0-250 μg/dose and most preferably, 0-100 μg/dose. MF59-0, therefore, refers to the above submicron oil-in-water emulsion lacking MTP-PE, while MF59-100 contains 100 μg MTP-PE per dose.

MF69, another submicron oil-in-water emulsion for use herein, contains 4.3% w/v squalene, 0.25% w/v Tween 80®, and 0.75% w/v Span 85® an optionally MTP-PE. Yet another submicron oil-in-water emulsion is SAF, containing 10% squalene, 0.4% Tween 80®, 5% pluronic-blocked polymer L121, and thr-MDP, also microfluidized into a submicron emulsion. Also useful with the present invention is the Ribi™ adjuvant system (RAS), (Ribi Immunochem, Hamilton, MT) containing 2% Squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of monophosphorylipid A (MPL), trehalose

dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL + CWS (Detox™).

For a description of various submicron oil-in-water emulsion formulations for use with the present invention, see, e.g., International Publication No. WO 90/14837; *Remington: The Science and Practice of Pharmacy*, Mack Publishing Company, Easton, Pennsylvania, 19th edition, 1995; Van Nest et al., "Advanced adjuvant formulations for use with recombinant subunit vaccines," In *Vaccines 92, Modern Approaches to New Vaccines* (Brown et al., ed.) Cold Spring Harbor Laboratory Press, pp. 57-62 (1992); and Ott et al., "MF59 -- Design and Evaluation of a Safe and Potent Adjuvant for Human Vaccines" in *Vaccine Design: The Subunit and Adjuvant Approach* (Powell, M.F. and Newman, M.J. eds.) Plenum Press, New York (1995) pp. 277-296.

It has also surprisingly been found that other adjuvants, in addition to submicron oil-in-water emulsions, administered prior to delivery of the gene of interest, also serve to enhance the immunogenicity of the antigen encoded by the gene. Such adjuvants include, but are not limited to: (1) aluminum salts (alum), such as aluminum hydroxide, aluminum phosphate, aluminum sulfate, etc.; (2) saponin adjuvants, such as Stimulon™ (Cambridge Bioscience, Worcester, MA), or particles generated therefrom such as ISCOMs (immunostimulating complexes); (3) Complete Freunds Adjuvant (CFA) and Incomplete Freunds Adjuvant (IFA); (4) cytokines, such as interleukins (IL-1, IL-2, etc.), macrophage colony stimulating factor (M-CSF), tumor necrosis factor (TNF), etc.; (5) detoxified mutants of a bacterial ADP-ribosylating toxin such as a cholera toxin (CT), a pertussis toxin (PT), or an *E. coli* heat-labile toxin (LT), particularly LT-K63 (where lysine is substituted for

the wild-type amino acid at position 63) LT-R72 (where arginine is substituted for the wild-type amino acid at position 72), CT-S109 (where serine is substituted for the wild-type amino acid at position 109), and PT-K9/G129 (where lysine is substituted for the wild-type amino acid at position 9 and glycine substituted at position 129) (see, e.g., International Publication Nos. WO93/13202 and WO92/19265); and (6) other substances that act as immunostimulating agents to enhance the immunogenicity of the antigen.

III. Experimental

Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperatures, etc.), but some experimental error and deviation should, of course, be allowed for.

Example 1

CTL Responses in Mice Immunized with Adjuvant Formulations Prior to Administration of HIV Plasmid DNA

BALB/c mice were divided into four treatment groups. One group, which received no adjuvant, served as a control. Group 2 was injected bilaterally with 50 μl of alum, mixed 1:1 with saline, in the tibialis anterior (TA) muscles. Group 3 was injected as above with MF59-0 (4.3% w/v squalene, 0.5% w/v Tween 80®, 0.5% w/v Span 85), mixed 1:1 with saline, and group 4 with MF59-100 (homogenization of a modified MF59 formulation containing 100 μg/dose MTP-PE), mixed 1:1 with saline. (See Van Nest et al., "Advanced adjuvant

formulations for use with recombinant subunit vaccines," in *Vaccines 92, Modern Approaches to New Vaccines* (Brown et al., ed.) Cold Spring Harbor Laboratory Press, pp. 57-62 (1992); and Ott et al., "MF59 -- Design and Evaluation of a Safe and Potent Adjuvant for Human Vaccines" in *Vaccine Design: The Subunit and Adjuvant Approach* (Powell, M.F. and Newman, M.J. eds.) Plenum Press, New York (1995) pp. 277-296).

Three days later, mice received a retroviral vector, HIV-IT (International Publication No. WO 91/02805; Ziegner, et al., AIDS (1995) 9:43-50). HIV-IT encodes the entire HIV-I_{IIIB} *env* gene, preceded by the first exon of the *rev* gene to facilitate HIV-I protein expression. 21 days following injection of the adjuvant, primed splenocytes were harvested, CTL were restimulated *in vitro*, and CTL activity assays were conducted.

Quantitative determination of CTLs was made using a limiting dilution assay, also known as CTL precursor frequency assay (CTLp), based on the single hit Poisson model as described by Taswell, *J. Immunol.* (1981) 126:1614-1619. The assays were conducted using methods well known in the art. Briefly, for limiting dilution assays, the *in vitro* restimulation step is done clonally, rather than in bulk splenocyte cultures (see Example 2, below). Sixty wells of a microculture plate were filled with a fixed number of primed splenocytes. Another set of 60 wells received a different number of primed cells; a third set of wells received a third number of cells; and so on. All wells also received irradiated target cells to stimulate any CTL in the well specific for the target antigen. After incubation for 7-10 days, aliquots of cells were transferred to wells containing radiolabelled target cells and ^{51}Cr release was

measured. Individual wells were scored either "+" or "-" for CTL activity, compared to the baseline release from wells containing labelled targets, but no effectors.

After tallying the numbers of wells containing CTL activity at each concentration of input primed splenocytes, the frequency of CTL in the input population can be calculated according to formulae set forth in Taswell, *J. Immunol.* (1981) 126:1614-1619. (Data presented here were calculated by the "minimum χ^2" method; calculations using the "maximum likelihood" method yielded nearly identical results.)

Table 1

Pretreatment	CTL Precursor Frequency
None	55,615 (45,968 - 70,388)
Alum	40,903 (35,333 - 48,557)
MF59-0	17,528 (14,810 - 21,468)
MF59-100	48,079 (40,009 - 60,227)

These numbers represent the inverse frequency of CTL among the primed splenocytes. In other words, in the spleens of animals preprimed with MF59-0, then immunized with HIV-IT, roughly 1 in 17,000 splenocytes are CTL specific for the antigens encoded by HIV-IT; whereas in animals which received no adjuvant, fewer than 1 in 55,000 splenocytes are HIV-IT specific CTL. Given in parentheses is the range of the 95% confidence interval for the frequency calculation, based upon the input data. The enhancement of CTL activity by MF59-0 pretreatment in this experiment is statistically significant; alum pretreatment shows a weaker enhancement of CTL activity; and MF59-100 does not appear to have any effect on this response.

Example 2

CTL Responses in Mice Immunized with Adjuvant Formulations Prior to Administration of HBV Retroviral Vector

C3H mice were divided into six treatment groups of three mice/group and injected with adjuvant (1:1 mixed with 140 mM NaCl) in the TA muscles, as described above. Two days later, mice received retroviral vector 6A3, either undiluted or diluted, in the same muscle sites. The following groups were included:

Control--undiluted vector
*Alum pretreatment; undiluted vector
*MF59-0 pretreatment; undiluted vector
Control--diluted vector (1:10)
Alum pretreatment; diluted vector (1:10)
MF59-0 pretreatment; diluted vector (1:10)

* due to sample contamination during restimulation, only 2 samples in each of these groups

Vector 6A3 is a retroviral vector that encodes a chimeric protein which is a fusion between the hepatitis B core protein, and the neoR protein. See, e.g., International Publication No WO 93/15207. Previous work has shown that this vector induces weak CTL responses in C3H mice.

21 days following injection of the adjuvant, spleens were harvested individually, and standard CTL activity assays were performed. Briefly, spleen cells from immunized BALB/c mice were cultured, restimulated, and assayed for CTL activity against ^{51}Cr-labelled target cells which expressed the HIV env/rev antigens and were thus susceptible to lysis by vector-induced CTL. Using known methods, target cells (T) were cultured with effector (E) cells at various E:T ratios for 4 hours. Aliquots of culture

supernatants were harvested, and the release of ^{51}Cr into the supernatants was quantitated by scintillation counting. 51Cr in the supernatants of experimental wells was compared with release from targets incubated without effectors ("spontaneous release"), and with targets incubated with detergent ("maximal release"). The "% specific release", a measure of CTL activity, is calculated using the formula:

$$\% \text{ specific release} = \frac{(\text{cpm released} - \text{spontaneous release}) \times 100}{(\text{maximal release} - \text{spontaneous release})}$$

The % specific release at varying E:T ratios is plotted in Figures 1A (undiluted vector) and 1B (diluted vector). As can be seen, MF59-0 significantly enhanced CTL induction, even when the vector was diluted such that little CTL activity was seen without the adjuvant pretreatment. It is interesting to note that two of three animals receiving diluted vector and MF59-0 pretreatment showed CTL responses stronger than animals receiving undiluted vector without pretreatment, suggesting that vector activity is enhanced 10-fold or more by MF59-0. Alum also showed an enhancing effect in this study; however, this effect was weaker than that seen with MF59-0.

b. A second study was performed in parallel with the above study. Here, C3H mice were given MF59-0 either one day prior or two days prior to injection with retroviral vector 6A3. CTL precursor frequencies were determined, as described in Example 1. Results are shown in Table 2.

Table 2	
Pretreatment	CTL Precursor Frequency
None	135,918 (119,508 - 157,552)
MF59-0 on day (-1)	46,667 (40,773 - 54,555)
MF59-0 on day (-2)	14,761 (11,586 - 20,337)

As in Table 1, results are presented as inverse frequency of CTL, with 95% confidence limits in parentheses. MF59-0 pretreatment significantly enhances the induction of CTL specific for 6A3 antigens; here, the effect is more pronounced when adjuvant is given 2 days prior to vector.

Example 3

IgG Induction in Mice Immunized with Adjuvant Formulations Prior to Administration of HIV-IT

Balb/c mice were divided into four treatment groups as described in Example 1 and administered alum, MF59-0 or MF59-100, each combined 1:1 with 140 mM NaCl, into the TA muscles, as described. Two days after adjuvant administration, mice were given the HIV-IT retrovirus vector as described in Example 1. At week nine, mice received a boost of HIV-IT vector without adjuvant.

Serum samples were collected prior to the first treatment, and at regular intervals thereafter, and levels of IgG1 specific for HIV gp120 determined using standard ELISAs. See, e.g., Fuller et al., *AIDS Res. Hum. Retroviruses* (1994) 10:1433-1441. The results are shown in Figure 2. Data are presented as the average of O.D. 450 for 1:100 serum dilutions. As can be seen, both alum and MF59-100 pretreatment enhanced Ig induction.

Accordingly, the use of submicron oil-in-water emulsions with nucleic acid immunization

techniques is disclosed. Although preferred embodiments of the subject invention have been described in some detail, it is understood that obvious variations can be made without departing from the spirit and the scope of the invention as defined by the appended claims.

We Claim:

1. Use of a submicron oil-in-water emulsion for the manufacture of a composition useful for nucleic acid immunization of a vertebrate subject wherein the immunization comprises administering a submicron oil-in-water emulsion to said vertebrate subject, and transfecting cells of said vertebrate subject with a recombinant vector comprising a nucleic acid molecule encoding an antigen of interest, under conditions that permit the expression of said antigen, thereby eliciting an immunological response to said antigen of interest in said vertebrate subject.

2. The use of claim 1, wherein said submicron oil-in-water emulsion is MF59.

3. The use of claims 1 or 2, wherein said submicron oil-in-water emulsion is administered prior to said recombinant vector.

4. The use of claims 1 or 2, wherein said submicron oil-in-water emulsion is administered subsequent to said recombinant vector.

5. The use of claims 1 or 2, wherein said recombinant vector is a nonviral vector.

6. The use of claims 1 or 2, wherein said recombinant vector is a viral vector.

7. The use of claim 6, wherein said viral vector is selected from the group consisting of a retroviral vector, a vaccinia vector and a canarypox virus vector.

8. The use of claims 1 or 2, wherein said vertebrate subject is a mammal.

9. The use of claim 8, wherein said mammal is a human.

10. The use of claims 1 or 2, wherein said antigen is a viral antigen.

11. The use of claim 10, wherein said viral antigen is a human immunodeficiency virus (HIV) antigen selected from the group consisting of an envelope glycoprotein and a gag protein.

12. The use of claim 11, wherein said HIV envelope glycoprotein is gp120, gp140 or gp160.

13. The use of claim 11, wherein said HIV gag protein is p24gag or p55gag.

14. The use of claim 10, wherein said viral antigen is a hepatitis antigen selected from the group consisting of hepatitis C virus (HCV) E2, HCV core antigen, hepatitis B virus (HBV) core antigen, HBV sAg, HBV pre-S1 and HBV pre-S2.

15. The use of claims 1 or 2, wherein said transfecting is done *ex vivo* and said transfected cells are reintroduced into said vertebrate subject.

16. The use of claims 1 or 2, wherein said transfecting is done *in vivo*.

17. A vaccine composition comprising a recombinant vector comprising a nucleic acid molecule

encoding an antigen of interest, and a submicron oil-in-water emulsion.

18. The vaccine composition of claim 28, wherein the submicron oil-in-water emulsion is MF59.

19. The vaccine composition of claims 17 or 18, wherein said antigen is a viral antigen.

20. The vaccine composition of claim 19, wherein said viral antigen is a human immunodeficiency virus (HIV) antigen selected from the group consisting of an envelope glycoprotein and a gag protein.

21. The vaccine composition of claim 20, wherein said HIV envelope glycoprotein is gp120, gp140 or gp160.

22. The vaccine composition of claim 20, wherein said HIV gag protein is p24gag or p55gag.

23. The vaccine composition of claim 19, wherein said viral antigen is a hepatitis antigen selected from the group consisting of hepatitis C virus (HCV) E2, HCV core antigen, hepatitis B virus (HBV) core antigen, HBV sAg, HBV pre-S1 and HBV pre-S2.

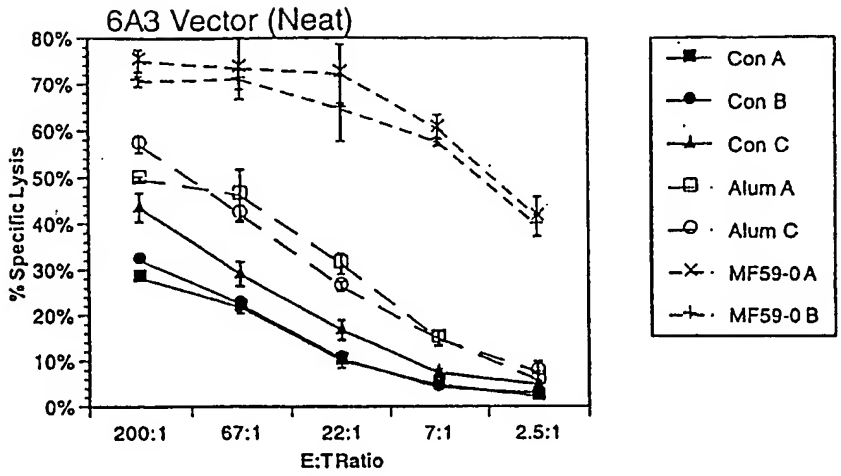
6A3 Vector (Neat)
% Specific Lysis
80%
70%
60%
50%
40%
30%
20%
10%
0%
200:1
67:1
22:1
7:1
2.5:1
E:T Ratio
Con A
Con B
Con C
Alum A
Alum C
MF59-0 A
MF59-0 B

FIG. 1A

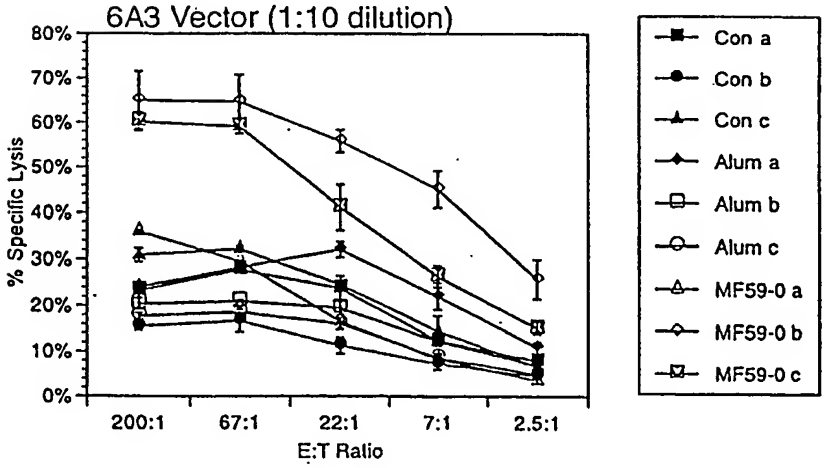
6A3 Vector (1:10 dilution)
% Specific Lysis
80%
70%
60%
50%
40%
30%
20%
10%
0%
200:1
67:1
22:1
7:1
2.5:1
E:T Ratio
Con a
Con b
Con c
Alum a
Alum b
Alum c
MF59-0 a
MF59-0 b
MF59-0 c

FIG. 1B

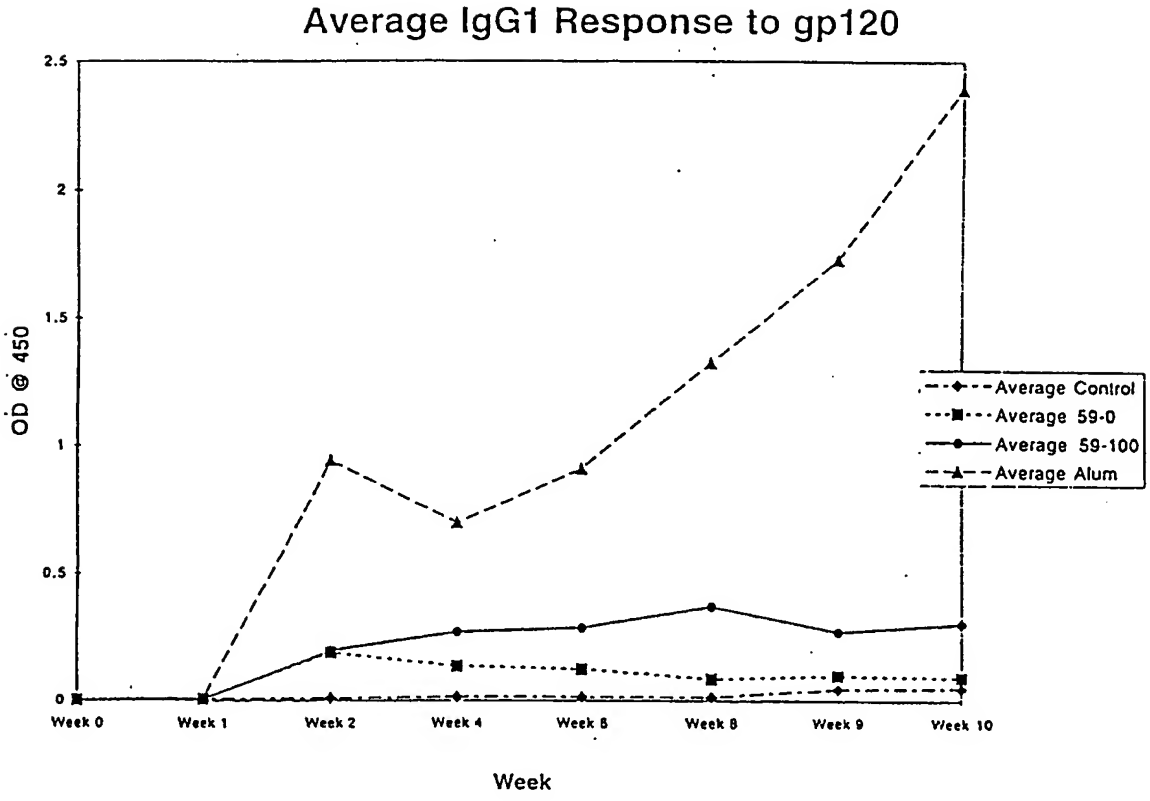
Average IgG1 Response to gp120
OD @ 450
2.5
2
1.5
1
0.5
0
Week 0
Week 1
Week 2
Week 4
Week 6
Week 8
Week 9
Week 10
Week
Average Control
Average 59-0
Average 59-100
Average Alum

FIG. 2

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(54) Title: USE OF SUBMICRON OIL–IN–WATER EMULSIONS WITH DNA VACCINES

(57) Abstract

The use of submicron oil–in–water emulsions with nucleic acid immunization techniques is disclosed. The method includes immunization with vaccine compositions containing nucleic acid molecules encoding one or more antigens of interest, as well as administration of a submicron oil–in–water adjuvant, such as MF59. The adjuvant can be administered either before, after or simultaneously with the nucleic acid vaccines.

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INTERNATIONAL SEARCH REPORT

Ir ıational application No.
PCT/US 98/14310

A. CLASSIFICATION OF SUBJECT MATTER

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Minimum documentation searched (classification system followed by classification symbols)

IPC6: A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 9416737 A1 (WEINER, DAVID, B.), 4 August 1994 (04.08.94), page 2, line 28 - page 13, line 27, page 25 - page 30, line 16, claims --	1-23
A	WO 9533835 A1 (CHIRON CORPORATION), 14 December 1995 (14.12.95), claims --	1-23
A	WO 9014837 A1 (CHIRON CORPORATION), 13 December 1990 (13.12.90), claims --	1-23

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
5 November 1998	11. 12. 98
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Anneli Jönsson

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/14310

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Dialog Information Services, file 155, MEDLINE, Dialog accession no. 08554776, Medline accession no. 96108867, Irvine KR et al: "Cytokine enhancement of DNA immunization leads to effective treatment of established pulmonary metastases"; & J Immunol (UNITED STATES) Jan 1 1996, 156 (1) p238-45	1-23

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INTERNATIONAL SEARCH REPORT

Information on patent family members

05/10/98

International application No.
PCT/US 98/14310

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		IL 108449 D	00/00/00
		JP 8509694 T	15/10/96
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